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Benzo(a)pyrene Metabolism in the America Oyster 'Crassostrea virginica'

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16. ABSTRACT

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A sensitive radioisotopic system was developed to permit the quantification of alkali-soluble and water-soluble BP metabolites produced by oyster mono-oxygenase. An NADPH- and O_2-dependent aryl hydrocarbon hydroxylase (AHH) was shown to be located in the digestive glands of these bivalves associated with the microsomal subcellular fraction. The specific activity of oyster AHH was considerably lower than that of laboratory mice, but was consistently demonstrable. The BP metabolites produced were primarily water-soluble derivatives.

There was some indication that oyster AHH was induced by chronic exposure of the animals to the environmental carcinogens BP and 3-methyl-cholanthrene. There was strong evidence that exposure to polychlorinated biphenyls (PCB) caused AHH induction.

High-pressure liquid chromatography was used to identify BP metabolites produced by oyster AHH. The generation of various dihydrodiol, quinone, and hydroxy BP derivatives was shown; this production was augmented in PCB-exposed oysters. This report covers the period from July 1, 1976 to June 30, 1977, and work was completed as of August 31, 1977.

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BENZO[a]PYRENE METABOLISM IN THE AMERICAN OYSTER *CRASSOSTREA VIRGINICA*

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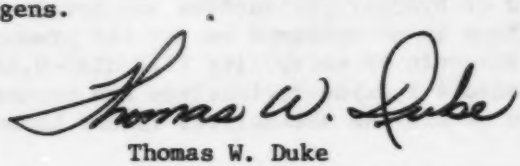
FOREWORD

The protection of our estuarine and coastal areas from damage caused by toxic organic pollutants requires that regulations restricting the introduction of these compounds into the environment be formulated on a sound scientific basis. Accurate information describing dose-response relationships for organisms and ecosystems under varying conditions is required. The Environmental Research Laboratory, Gulf Breeze, contributes to this information through research programs aimed at determining:

- the effects of toxic organic pollutants on individual species and communities of organisms;
- the effects of toxic organics on ecosystem processes and components;
- the significance of chemical carcinogens in the estuarine and marine environments.

The present report deals with a subject of great potential significance in the area of early detection of carcinogens in the environment. The action of carcinogens on enzyme systems of aquatic species should provide the basis for sensitive early warning detection tests. Further, the susceptibility of aquatic species to carcinogens or mutagens may depend on the ability of these species to metabolize xenobiotics to proximal or active intermediate compounds. This report provides data on the metabolic responses of oysters to known carcinogens and mutagens.



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A sensitive radioisotopic system was developed to permit the quantification of alkali-soluble and water-soluble BP metabolites produced by oyster mono-oxygenase. An NADPH- and O_2-dependent aryl hydrocarbon hydroxylase (AHH) was shown to be located in the digestive glands of these bivalves associated with the microsomal subcellular fraction. The specific activity of oyster AHH was considerably lower than that of laboratory mice, but was consistently demonstrable. The BP metabolites produced were primarily water-soluble derivatives.

There was some indication that oyster AHH was induced by chronic exposure of the animals to the environmental carcinogens BP and 3-methylcholanthrene. There was strong evidence that exposure to polychlorinated biphenyls (PCB) caused AHH induction.

High-pressure liquid chromatography was used to identify BP metabolites produced by oyster AHH. The generation of various dihydrodiol, quinone, and hydroxy BP derivatives was shown; this production was augmented in PCB-exposed oysters. Quinones were the major metabolites in normal oysters; the production of hydroxy derivatives was particularly stimulated by PCB induction. There is no evidence as yet for production of the suspected ultimate carcinogenic BP metabolite (7,8 diol-9,10-epoxide); the 7,8-diol and the mutagenic 4-5 oxide derivatives are present in the oyster. However, identification of all the metabolites is not known.

This report was submitted in fulfillment of Grant No. R804435 by the Sloan-Kettering Institute for Cancer Research under the partial sponsorship of the U. S. Environmental Protection Agency. This report covers the period from July 1, 1976 to June 30, 1977, and work was completed as of August 31, 1977.

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SECTION 1

INTRODUCTION

As a result of industrialization, a wide variety of xenobiotics is constantly introduced into the environment. One of the most ubiquitous of these pollutants is the carcinogenic polycyclic aromatic hydrocarbon benzo-[a]pyrene (BP). It is thought that certain BP oxide metabolites are responsible for its mutagenicity and carcinogenicity. These BP derivatives are produced by aryl hydrocarbon hydroxylase (AHH), which is associated with the smooth endoplasmic reticulum of hepatic cells in mammals and digestive gland cells in invertebrates.

Filter-feeding mollusks are known to concentrate BP and other hydrocarbon pollutants of their aquatic environment. A considerable portion of these hydrocarbons can be eliminated by depuration; however, the question of BP metabolism by mollusks has not been thoroughly studied. It is important to assess the capacity of marine mollusks to metabolize BP as a step in our understanding of invertebrate chemical carcinogenesis; also, the presence of potentially carcinogenic metabolites in shellfish may have public health implications. We have developed a sensitive in vitro method to quantify the production of radiolabeled BP derivatives by oyster AHH. The results of these studies are included in this report.

We present evidence that oyster AHH is inducible by commercial polychlorinated biphenyl mixtures (PCB's), which represent an important class of environmental contaminants. If future work proves that bivalve AHH activity levels are directly proportional to the levels of pollutants to which the animals are exposed, it might be possible to develop a sensitive bio-indicator system for monitoring environmental pollution.

Aryl hydrocarbon hydroxylase catalyzes the production of a variety of biologically active and inactive BP metabolites. It is essential to identify the BP derivatives actually generated under particular conditions in order to speculate on carcinogenicity. In the present study, high-pressure liquid chromatography was used to resolve and tentatively identify the BP metabolites generated by AHH from normal and PCB-exposed oysters.

SECTION 2

CONCLUSIONS AND RECOMMENDATIONS

Technical difficulties have been resolved for development of an assay system to measure aryl hydrocarbon hydroxylase (AHH), using the carcinogenic substrate benzo[a]pyrene (BP) in the oyster Crassostrea virginica. It is possible to show a low, but consistent, level of AHH activity in oysters. This activity is primarily reflected in the production of water-soluble metabolites, although other more nonpolar (NaOH-soluble) metabolites are also produced. The enzyme is O_2- and NADPH-dependent, and greatest specific activity is associated with the microsomal subcellular fraction of digestive gland homogenates. Whereas some information concerning cofactor requirements, temperature and pH optima, and enzyme kinetics is now known, some very basic facts need to be determined. For example, although cytochromes play a major role in AHH reactions in higher animals, their involvement in oyster BP metabolism has not been studied. The comparatively high percentage of water-soluble BP metabolites produced suggests that conjugation of metabolites to sulfate, glucuronic acid, etc. is an important pathway of BP metabolism in oysters; sulfotransferase and other conjugating enzymes have not as yet been studied.

There is strong evidence that oyster AHH is readily inducible by polychlorinated biphenyls (PCB's); PCB's are quite stable and are major environmental pollutants in many geographical areas. This observation should be followed up; it is important to know such parameters of the inductive response as dose-dependency, duration, and influence degree of chlorination and isomeric composition of the various commercial PCB mixtures. If indeed AHH activity can be shown to be directly proportional to pollutant concentrations in the aqueous environment, it might be possible to use its level of activity in shellfish as a sensitive bioindicator of pollution. Laboratory studies should be coordinated with field studies of AHH levels in mollusks collected from waters of varying, known concentrations of contamination.

The ability of oysters to metabolize BP has now been established. Such oncogen metabolism is thought to be required to produce active derivatives that are able to react covalently with biological macromolecules such as DNA. This alkylation reaction is probably responsible for mutagenicity and carcinogenicity of BP. Both active and inactive (detoxified) BP metabolites are produced; therefore, it is necessary to identify the specific metabolites generated by oyster AHH. We have separated six major BP metabolites produced by the oyster, using high-pressure liquid chromatography. These include dihydrodiols, quinones, and hydroxylated derivatives. Most of these

metabolites are probably poor carcinogens, based on their lack of mutagenicity for bacteria and mammalian cells. However, the 7,8-dihydrodiol may be present; this is the precursor for the suspected ultimate carcinogen, which is the 9-10 epoxide of this compound. Also, there is evidence for the presence of the 4,5-oxide metabolite which is moderately mutagenic in several assay systems. It is very important to establish definitively the identity of each of the BP metabolites produced by oyster AHH; an indication of this can be obtained by coelution studies using known metabolite standards. Those metabolites not commercially available can be obtained through the generosity of our collaborators. Furthermore, these metabolites should be tested for mutagenicity with the Ames bacterial system.

SECTION 3

EXPERIMENTAL PROCEDURES

GENERAL PROCEDURES

The presence of aryl hydrocarbon hydroxylase (AHH) in oyster tissues was determined *in vitro* by measuring the formation of labeled derivatives of 7, 10-^{14}C-benzo[a]pyrene (BP) during incubation with homogenates of microsomes. Mixed-function oxidases, including AHH, require the cofactor NADPH (nicotinamide adenine dinucleotide phosphate, reduced form) and oxygen. Oyster AHH also conforms to these requirements; however, it was soon shown that cofactor concentrations optimal for mammalian systems were inappropriate for the study of molluscan enzymes. For example, the NADPH concentration recommended by Nebert and Gelboin (1) is inhibitory for oyster AHH but excellent for use with preparations from laboratory rodents.

The reaction is stopped after incubation of enzyme and substrate under appropriate conditions, and the reaction mixture is extracted by using a modification of the method of Abramson and Hutton (2). The parent compound and a number of metabolites are extracted in hexane; the metabolites are separated from BP by additional extraction with NaOH. Other metabolites remaining in the aqueous phase include water-soluble derivatives and metabolites that have been conjugated with glucuronic acid and/or sulfate. Both NaOH-soluble and water-soluble metabolites are quantified by using a liquid scintillation spectrometer.

ENZYME PREPARATION

Fresh oysters (*Crassostrea virginica*) were opened, and the visceral mass excised and immediately placed on ice. The digestive gland was dissected as free as possible from surrounding tissues and a 25% by weight homogenate prepared in ice-cold 0.839 M sucrose. Homogenates were first centrifuged at 10,886 g for 10 min in a Sorvall RC2-B automatic refrigerated centrifuge using an SS-34 rotor. Homogenate supernatants were recentrifuged at 10,886 g. The resulting supernatants were ultracentrifuged at 100,000 g in a Beckman Model L preparative ultracentrifuge using a 40 rotor. The microsomal pellets were resuspended in 0.839 M sucrose at 1°C. All digestive gland homogenates and/or microsomes were held in an ice bath until the enzyme assays were performed; this period never exceeded 30 min. An aliquot of each homogenate or microsomal preparation was frozen for subsequent protein determination by the method of Lowry et al. (3).

ENZYME ASSAY

The incubation conditions described here were found to be optimal for the demonstration of AHH activity in oyster digestive gland. Because no procedure for measuring AHH activity in bivalves exists in the literature, considerable effort was expended in determining the optimal cofactor concentrations, time, temperature, buffer system, etc.

One milliliter reaction mixtures were prepared in 10-ml Erlenmeyer flasks and held on ice until the incubation stage. The final concentrations of the components of the reaction mixture were 0.1 mM NADPH (reduced nicotinamide adenine dinucleotide phosphate), 5 mM $MgSO_4$, and 1.6 mg/ml albumin (bovine serum albumin, fraction V) in 0.4 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid) buffer, pH 7.8. These compounds were purchased from the Sigma Chemical Company, St. Louis, Missouri. Digestive gland homogenate (0.3 ml) or microsomes (0.05 ml) then were added to each flask, and the reaction was initiated by the addition of 80 nmoles (0.1 µCi) 7,10-^{14}C-benzo-[a]pyrene, purchased from Amersham/Searle Corporation, Arlington Heights, Illinois. The desired amount of ^{14}C-BP was introduced into the reaction mixture dissolved in 0.025 ml acetone; this amount of acetone had no effect on AHH activity.

Experimental flasks were incubated 60 min at 30°C in an Eberbach shaking water bath; the reaction was stopped by the addition of 1 ml cold acetone. Reactions in control (T_o) flasks were stopped immediately after the addition of ^{14}C-BP. Once the reactions were terminated, both T_{60} and T_o samples were subjected to the same methodological protocols. Reaction mixtures were extracted with hexane 15 min at 30°C in a shaking water bath. A 1-ml aliquot of the hexane layer was removed and vigorously mixed with an equal volume of 1N NaOH; a 0.5 ml sample of the NaOH extract was placed in a scintillation vial. Additional NaOH extractions of the hexane layer showed that all of the NaOH-soluble metabolites had been removed in the initial extract. The aqueous layer was further extracted with 2 ml of hexane to assure that all the labeled parent compound had been eliminated. A 0.5 ml aliquot of the aqueous layer was pipetted into a scintillation vial; the protein in this sample was solubilized by overnight incubation at room temperature in 0.25 ml Protosol (New England Nuclear, Boston, Massachusetts).

The radioactivity in the NaOH and aqueous aliquots was quantified by using a Packard Model 3255 liquid scintillation spectrometer. Appropriate control (T_o) values were subtracted from experimental (T_{60}) results. The total amount of radioactivity, representing total metabolites produced, in the NaOH and aqueous layers from a given sample was calculated, taking into account quenching, machine efficiency, partitioning, and dilution. The nmoles of ^{14}C-BP metabolized during the course of the experiment were determined based on the specific activity of the parent compound. The activity of benzo-[a]pyrene hydroxylase was expressed as nmoles ^{14}C-BP metabolized/mg protein/60 min.

SECTION 4

RESULTS

OPTIMAL ASSAY CONDITIONS

The experimental conditions given in the procedure for AHH assay were determined by measuring the influence of several factors on the enzymatic reaction *in vitro*. Some of those studies are summarized below.

Cofactor Concentration

The cofactor NADPH is required for AHH activity. In this system the presence of 0.1 mM NADPH usually enhanced AHH activity; however, it should be noted that considerable activity was measured in homogenates in the absence of added NADPH. This presumably was due to the natural presence of the cofactor in homogenate. NADPH requirement was most easily shown with washed microsome preparations. The addition of NADPH in excess of 0.5 mM severely inhibited the metabolism of BP by oyster AHH. Various concentrations of NADH (nicotinamide adenine dinucleotide, reduced form) failed to alter the rate of reaction; it would appear that NADPH is the preferred electron donor. The addition of an NADPH-generating system composed of NADP, glucose-6-phosphate, and glucose-6-phosphate dihydrogenase was shown not to enhance AHH activity. Results from a series of experiments using various concentrations of each of these components indicated that the generating system was not useful in this system.

Temperature

The incubation temperature selected for these studies was 30°C. This temperature, while higher than physiological for these poikilothermic animals, was shown to favor the activity of BP hydroxylase. However, it was noted that considerable enzymatic activity could be demonstrated at 15°C and 22°C. A temperature of 30°C is reached in the estuaries of the Gulf Coast during July and August. The data indicate that the enzyme is heat-stable at temperatures in excess of those likely to exist *in vivo*. In a typical series of four determinations at each temperature, the following mean AHH-specific activities ± SD (standard deviation) were found: 0°, 0.063 ± 0.008; 15°, 0.301 ± 0.21; 22°, 0.348 ± 0.030; 30°, 0.422 ± 0.052; 37°, 0.219 ± 0.056. Little BP hydroxylase activity was measured at temperatures above 60°C.

Oxygen Requirement

Oxygen dependency of oyster AHH was shown by N_2 inhibition of enzyme activity. Nitrogen was bubbled through the reaction mixture for 3 min prior to the addition of ^{14}C-BP. Subsequently, the reaction vials were gassed with N_2, capped, and incubated following the standard protocol. Untreated samples were run simultaneously as controls. Benzo[a]pyrene metabolism was inhibited 36.3 ± 12.5% (n = 4) in the N_2-treated aliquots as compared to identical untreated homogenates. It is probable that some O_2 remained dissolved in the medium after gassing with N_2, permitting the reaction to proceed at a reduced rate.

Dithiothreitol

It has been shown that maximal mammalian AHH activity is obtained when DTT (DL-dithiothreitol) is present in medium (2). This reagent is useful for maintaining -SH groups in the reduced state (4). Accordingly, we assayed the effect of 1-10 µmoles/ml DTT on Crassostrea BP hydroxylase activity. The procedure resulted in slight, unpredictable fluctuations in activity; therefore, we abandoned its use in these studies.

BASELINE AHH ACTIVITY IN OYSTERS

The activity of benzo[a]pyrene mono-oxygenase in normal Crassostrea virginica digestive gland homogenate, as measured by the assay described above, was 0.465 ± 0.128 nmoles/mg protein/60 min (n = 20). This represents the sum of the NaOH-soluble BP metabolites (0.041 ± 0.039) and the water-soluble BP metabolites (0.424 ± 0.104). The removal of the nuclei and cell debris from the homogenate by low-speed centrifugation (11,000 g) only slightly reduced total AHH activity to 0.436 ± 0.082 (n = 5). It was shown by further centrifugation (100,000 g) that the activity was primarily associated with the microsomal fraction, the AHH specific activity of which was at least twice that of the whole digestive gland homogenate.

TIME-COURSE OF BP METABOLISM

After an initial burst of oyster AHH activity, there is a steady production of BP metabolites for at least 60 min in this system. The rate of reaction is constant during this period (Figure 1); therefore, 60 min was selected for the length of incubation. The T_{60} sample contains comparatively high amounts of BP metabolites, and 60 min is on the linear portion of the curve. The data are best represented by a straight line because the correlation coefficient equals 0.992.

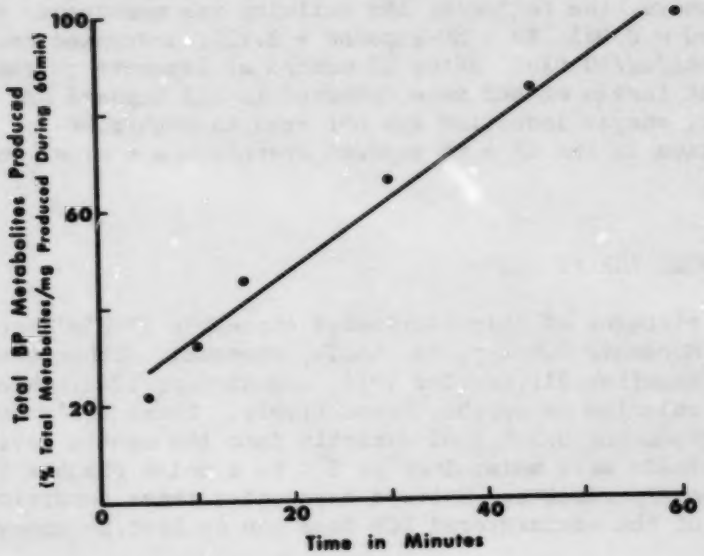

Figure 1. The production of BP metabolites by oyster homogenate during 60 min incubation. Values are expressed as percentage of total metabolites generated in 60 min; mean values of two experiments are indicated. Line was produced by calculating least-squares linear regression.

INDUCTION OF OYSTER AHH BY CARCINOGENS

As a part of a study of the oyster as an indicator of environmental carcinogens (5), exposed oysters were periodically sent to this laboratory for AHH analysis. These animals had been exposed to 1.0 µg/l BP, or 3-methylcholanthrene (MC), or a combination of both carcinogens. The usual exposure regimen was 8 hr/day, 5 days/week. Representative carcinogen-exposed and control oysters were periodically quick-frozen and shipped in dry

ice for AHH assay. All data reported in this section are the means of AHH activity in two animals from each group.

After 5 months of exposure, the following AHH activity was measured in digestive gland homogenates: BP-exposed = 0.183 nmoles BP products/mg/10 min; MC-exposed = 0.206; unexposed control animals had no detectable activity. These data suggest possible AHH induction by the carcinogens. The comparatively low apparent enzyme specific activity may be explained by the short incubation period (thought to be optimal at the time of these assays) and by the fact that some AHH activity is lost during freezing and thawing. After 9 months of exposure, the following AHH activity was measured: BP-exposed = 0.107; MC-exposed = 0.081; BP + MC-exposed = 0.121; unexposed control = 0.035 nmoles BP products/mg/10 min. After 13 months of exposure to these carcinogens, significant levels of AHH were detected in all exposed and control animals; however, enzyme induction was not seen in either BP- or MC-exposed animals. Induction in the BP + MC-exposed oysters was minimal and probably not significant.

INDUCTION OF OYSTER AHH BY PCB's

Commercial mixtures of polychlorinated biphenyls (PCB's) were kindly supplied by the Monsanto Company, St. Louis, Missouri. Those used in this study included Capacitor 21, Aroclor 1016, and Aroclor 1242, which contain 21, 41, and 42% chlorine by weight, respectively. These PCB's were applied topically by introducing 0.3-0.5 ml directly into the mantle cavity of the oysters. The animals were maintained at 5°C in a moist chamber for the duration of these experiments. Oysters kept under these conditions are viable and none of the administered PCB dose can be lost by exposure to water.

Preliminary results indicate that oyster AHH, like mammalian MFO, is inducible by PCB's. Administration of Aroclor 1242 produced an approximate 2-fold (2.29 ± 0.6, n = 5) increase in AHH activity by 48 hr. Similar levels of induction were produced by Capacitor 21 (218% increase in activity, n = 4) and Aroclor 1016 (304% increase, n = 4).

RESOLUTION OF OYSTER BP METABOLITES BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

Recently, a technique for efficient separation of BP metabolites using high-pressure liquid chromatography (HPLC) has been developed by Selkirk et al. (6). In collaboration with Dr. George B. Brown of this Institute, we applied this method to separate BP metabolites produced by oyster AHH.

Flasks containing 1 ml reaction mixtures, each containing 0.3 ml oyster digestive gland homogenate, albumin, NADPH, etc. (as described above), were prepared. The reaction was initiated by the addition of 80.5 nmoles (0.625 μCi) ^{3}H-BP; the use of higher specific activity BP permitted more accurate quantification of metabolites. Each run consisted of 10 T_o and 10 T_{60} flasks. At the appropriate time the reactions were terminated by the addition of 1 ml cold acetone, and each flask was extracted with 2 ml ethyl

acetate. The ethyl acetate layers from the T_o flasks were pooled with each other; the ethyl acetate layers from the T_{60} flasks were also combined in a separate pool. Anhydrous $MgSO_4$ (1.0 g) was added to each pooled extract and filtered out, after vigorous mixing. The ethyl acetate samples were dried under N_2 and redissolved in 0.2 ml absolute methanol. The samples were injected into a 2.1 x 1000 mm permaphase (1-m ODS) column. The column was eluted with a reverse phase gradient system with methanol and water (30:70 initially). The gradient rate of change was 3%/min; column temperature was 45°C; the pressure was about 600 pounds/in^2; flow rate was 0.6 ml/min. Ultraviolet absorption was continuously monitored, and samples from the fraction collector were assayed for radioactivity by liquid scintillation spectrometry.

Peaks corresponding to the major classes of BP metabolites produced by mammalian AHH were also produced by oyster BP hydroxylase. The identification of metabolites was made by comparing Rfs of unknown peaks with those of known standards. This information is summarized in Table 1.

TABLE 1. BP METABOLITES PRODUCED BY OYSTER AHH, RESOLUTION BY HPLC

Peak number*	Rf†	Probable identity
1	0.059 ± 0.015	9,10-dihydrodiol
2	0.175 ± 0.013	4,5-dihydrodiol (or 7,8-dihydrodiol)
3	0.500 ± 0.028	1,6-quinone (or 3,6-quinone)
4	0.668 ± 0.034	3-hydroxybenzo[a]pyrene
5	0.794 ± 0.022	Unknown monohydroxylated derivative
6	1.000	Benzo[a]pyrene

* Peaks common to all oyster preparations analyzed

† Mean Rf ± SD, n = 18

The exact identification of many of these metabolites awaits coelution experiments with known standards. However, as indicated, these radiolabeled metabolites are consistently seen in all oyster preparations after incubation with ^{3}H-BP. Furthermore, the peaks are distinct and do not overlap. Although there is some uncertainty concerning their precise identity, it may be said that peaks 1 and 2 represent dihydrodiols; peak 3 represents quinones, and peaks 4 and 5 are hydroxylated BP metabolites.

The percentage increase observed in each of these metabolites was measured by comparing T_{60} with T_o results. Whereas the actual percentages of

of increase varied considerably, there was general production of all 5 major metabolites with time, as shown in Table 2. In control oysters, the quinones were the major metabolites produced, followed by the dihydrodiols and the hydroxylated products. The metabolite profiles in control oysters were compared to those in oysters 24 hr after exposure to Aroclor 1242, according to the protocol described above. In AHH-induced oysters, again the quinones were the major metabolites, followed by the hydroxy- and dihydrodiol metabolites. Aroclor treatment produced AHH induction as evidenced by an increased production of BP metabolites. After 60-min incubation of oyster AHH and ^{3}H-BP, metabolites accounted for 1.196% of the total radioactivity present in ethyl acetate extracts. This percentage increased to 2.205% in the case of Aroclor-exposed oysters. It appeared that the production of hydroxy derivatives and quinones is more pronounced in PCB-induced oysters. It must be remembered that this procedure measures only those BP metabolites that are soluble in organic solvents.

TABLE 2. PERCENT TOTAL CPM's IN BP METABOLITES FROM CONTROL AND AROCLOR 1242-INDUCED OYSTERS

Peak number	Control	Induced
1	0.198 ± 0.205	0.264 ± 0.045
2	0.266 ± 0.299	0.125 ± 0.093
3	0.520 ± 0.602	1.095 ± 0.417
4	0.098 ± 0.097	.318 ± 0.635
5	0.114 ± 0.163	.403 ± 0.488
6	98.804	97.795

SECTION 5

DISCUSSION

Microsomal MFO activity has been described in many vertebrates and in various lower animals. This enzyme system represents a major pathway for detoxification of a host of xenobiotic chemicals. This paper presents, for the first time, evidence for this enzyme system in a marine mollusk Crassostrea virginica. Aryl hydrocarbon hydroxylase activity was measured in the oyster by a sensitive radioisotopic technique, as well as by high-pressure liquid chromatography. Since other investigators have not demonstrated the presence of AHH in mollusks, it is important to explain these findings by comparing the methodologies used in the various assays.

METHODOLOGICAL CONSIDERATIONS

Most laboratories report the metabolism of BP in terms of fluorescent equivalents, in which the results are compared to the fluorescence of 3-OH-BP which is described as the major metabolite. This assay tells nothing of the total number or the concentration of the metabolites produced; it only shows that one or more of the metabolites formed have a certain fluorescence. This method is not as sensitive as the method described in this paper, in which the production of both organic solvent-soluble and water-soluble ^{14}C-BP metabolites is quantified. Since nothing was known about the BP metabolite profile in oysters, the usefulness of a method which depended heavily upon the production of 3-OH-BP was considered questionable.

A major difficulty in demonstrating oyster AHH resulted from the use of an excellent assay system developed for mammalian cells (1) for similar assays in bivalves (7). It would be unlikely that the ideal medium composition for mammalian cells would be useful in work with oysters. Indeed, this work shows that oyster AHH is effectively inhibited by NADPH concentrations commonly used in mammalian MFO studies. As already mentioned, it was necessary to modify the salt composition, buffer system, and the strength of the buffer to optimize conditions for oyster AHH.

Several other reasons can be suggested to explain the apparent lack of AHH in bivalves reported in the literature. The use of whole animal homogenates makes detection of AHH activity unlikely. In oysters, AHH activity is high only in the digestive gland, as in the case of mammals where activity is predominantly found in the liver. In whole animal homogenates, digestive gland tissue is diluted with all the other components of the organism. In such preparations AHH is exposed to proteolytic digestive enzymes, which

would probably denature it. In addition, AHH could be exposed to endogenous MFO inhibitors, such as those associated with the gut contents and certain natural pigments in other invertebrates (8,9). Furthermore, most studies of polycyclic aromatic hydrocarbon (PAH) metabolism concentrate only on quantifying metabolites that are soluble in organic solvents; the water-soluble metabolites are ignored. Our data indicate that the majority of BP metabolites produced by the oyster are water-soluble.

THE FATE OF PAH IN BIVALVES

There is no doubt that bivalves can remove from seawater and concentrate within their tissues a variety of PAH. In an early study, Zechmeister and Koe (10) showed that barnacle extracts contained PAH, including BP, which probably originated "from tarry materials floating along the southern California coast." The ability of mussels to take up environmental hydrocarbons is well established (11,12); it has been suggested that these animals could be useful in estimating PAH contamination in the marine environment (5,13,14). It has been generally held that bivalves are able to dispose of much of the hydrocarbon load by depuration processes, but are not able to metabolize PAH by mixed-function oxygenase enzymes (7,15-18).

However, there was a report, prior to this one, that indicated that mollusks have some MFO activity. Mollusks can synthesize sterols (19), a process in which squalene is converted into the 2,3-oxide as an intermediate via a microsomal NADPH-dependent oxidase (20).

COMPARISON OF AHH ACTIVITY IN OYSTERS AND OTHER ANIMALS

The basic similarity between the characteristics of AHH in oysters and other animals has already been described in this paper. In this section the level of AHH activity in oysters is compared to that in another invertebrate and laboratory mice. This work was done in the laboratory using the same technique for separating BP metabolites; however, the conditions for the reaction were optimal for each species studied.

As indicated in Table 3, AHH activity can be consistently recorded in the oyster. However, the level of activity is only about 1/13 of that seen in laboratory mice and also is lower than that reported for an insect (21). The NaOH-extracted metabolites comprise less than 1/10 of the total BP metabolites produced by oyster AHH. This is not the case for the other animals studied, in which about equal quantities of metabolites were recovered from the water and NaOH phases.

TABLE 3. COMPARISON OF AHH ACTIVITY* IN VARIOUS ANIMALS AS DETERMINED BY THE GENERATION OF RADIOISOTOPIC BP METABOLITES

Animal	n	Total activity†	Water-soluble derivatives	NaOH-soluble derivatives
Mouse ♂ C57BL/6	8	2.12	1.05 ± 0.24	1.07 ± 0.18
Mouse ♂ Camm Swiss-Webster	8	2.37	1.32 ± 0.37	1.05 ± 0.30
Insect *Spodoptera eridania*	15	0.398	0.169 ± 0.044	0.229 ± 0.131
Oyster *Crassostrea virginica*	20	0.163	0.149 ± 0.036	0.014 ± 0.013

* AHH activity in uninduced, normal digestive gland (or liver) homogenates.

† nmoles BP metabolites/mg protein/10 min.

TENTATIVE IDENTIFICATION OF BP METABOLITES PRODUCED BY OYSTERS

It is thought that metabolic activation of PAH, such as BP, is required before they can express biological activity. BP does not bind covalently to biological macromolecules, including DNA; its active intermediates are capable of this interaction, which probably results in subsequently observed mutagenicity and carcinogenicity. This metabolic activation is mediated by AHH, which also catalyzes the detoxification of oncogens. The final physiological result of BP metabolism depends on the balance between the detoxifying and activating pathways, as well as on target tissue sensitivity.

The primary BP metabolites are epoxides (22). BP epoxides are transformed to phenols by spontaneous rearrangement, to dihydrodiols by epoxide hydrase, to glutathione conjugates by GSH-epoxide-transferase, or to derivatives that are bound covalently to tissue macromolecules. Phenolic derivatives can be further conjugated with glucuronic acid or sulfate. Dihydrodiols may be converted to diol-epoxides via AHH and cytochrome P-450 or P-448.

The susceptibility of invertebrates to chemical carcinogens is largely unknown. An aim of this study was to determine if the potent mammalian carcinogen and environmental contaminant BP could be considered as a possible bivalve carcinogen. To this end, the ability of the oyster to metabolize BP was shown in a sensitive radioisotopic assay. Next an attempt was made to identify specific BP metabolites using high-pressure liquid chromatography. Aside from possible carcinogenic effects on the oyster, the presence of carcinogenic BP metabolites in shellfish, common in the human diet, might have public health implications. It has been suggested tentatively that a correlation may exist between PAH pollution and neoplasia in certain bivalves (23, 24).

The vast literature on the cytotoxicity, mutagenicity, and carcinogenicity of BP derivatives cannot be reviewed here. However, it is generally thought that epoxides, produced via the NADPH-dependent microsomal monooxygenases, are the important reactive metabolites responsible for carcinogenesis. This idea was supported by studies of the properties of synthetic K-region epoxides, which are alkylating agents that bind covalently to nucleic acids. However, more recent work suggests that diol-epoxides, particularly 7,8-dihydro-7,8-dihydroxybenzo[a]pyrene 9,10-oxide, are the most likely carcinogenic forms of BP (25). These epoxides would be in the form of the corresponding dihydroxydiols in the chromatographed extracts, due to the action of epoxide hydrase. The ultimate carcinogenic BP metabolite would be tetradiol, which would chromatograph with a retention time less than the first diol to appear (9,10-diol-BP). Recently, 29 BP derivatives were tested for mutagenic activity against bacterial cells (the Ames assay) and mammalian cells (Chinese hamster V79) by Wislocki et al. (26). BP-4,5-oxide was the most mutagenic compound tested in this report, but BP-7,8-diol-9,10-epoxide was shown to be 40-60 times more mutagenic and cytotoxic than BP-4,5-oxide (27). All the other arene oxides, phenols, quinones and dihydrodiols were not (or weakly) mutagenic, with the exception of BP- 1,12-oxide, and 6-OH BP mutagenic to hamster cells, and 6-OH BP and 12-OH BP moderately mutagenic for bacteria.

According to our interpretation of HPLC separation of BP metabolites produced by oyster AHH, there is little evidence as yet for the production of highly mutagenic or carcinogenic BP derivatives. The first labeled peak in these chromatographs corresponds to the 9,10-diol. The most suspect carcinogenic BP derivative should have a shorter retention time; however, there is no indication of such a peak in these preparations. Both normal and Aroclor-induced oysters produce BP-4,5-oxide which is somewhat mutagenic for both bacteria and mammalian cells. The production of BP-4,5-oxide is apparently not induced by PCB exposure. The major BP metabolites produced by oyster AHH are quinones, which are rather inactive. Hydroxy BP derivatives (such as 3-OH BP), which are generated particularly in PCB-induced oysters, are generally inactive. However, there is evidence for the mutagenicity of certain other hydroxy BP metabolites, and peak 5 contains an unidentified monohydroxylated derivative.

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